Articles

Role of Nitric Oxide in Progression and Regression of Atherosclerosis

JOHN P. COOKE, MD, PhD, *Stanford, California*

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Endothelium-derived nitric oxide is a potent endogenous vasodilator that is derived from the metabolism of L-arginine. This endothelial factor inhibits circulating blood elements from interacting with the vessel wall. Platelet adherence and aggregation as well as monocyte adherence and infiltration are opposed by this paracrine substance. By virtue of these characteristics, endothelium-derived nitric oxide inhibits atherogenesis in animal models and may even induce regression.

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The endothelium of the blood vessel wall is a diaphanous tissue only one cell layer thick. Despite its delicate appearance, the endothelium is a potent regulator of vascular tone and structure by virtue of the panoply of paracrine factors it produces. One of these paracrine substances is endothelium-derived relaxing factor (EDRF). The seminal article reporting the discovery of EDRF was published in 1980 by Furchgott and Zawadski, who speculated that it was a prostanoid metabolite of lipoxygenase.[1] Endothelium-derived relaxing factor is now known to be nitric oxide (NO).[2,3] In physiologic conditions, this molecule has a short half-life (measured in fractions of a second) unless it is bound to a carrier molecule, such as a thiol. Indeed, evidence indicates that EDRF may be released in the form of a nitrosothiol.[4] Nitric oxide is derived from the activity of the enzyme NO synthase, which oxidizes L-arginine to yield L-citrulline and NO.[5] Nitric oxide is the most potent endogenous vasodilator known and exerts its actions in the same manner as other nitrovasodilators such as nitroglycerin.[6] The NO moiety of nitrovasodilators activates soluble guanylyl cyclase within the vascular smooth muscle, leading to the production of cyclic guanosine monophosphate (cGMP). This cyclic nucleotide is the second messenger for the action of NO, and it activates cGMP-dependent proteins that mediate vascular smooth muscle relaxation.

Nitric oxide is released when specific receptors on the endothelium are occupied by their respective agonists, many of which circulate in the bloodstream. These agonists include thrombin and adenosine diphosphate released by aggregating platelets.[7-9] When the endothelium is intact, adenosine diphosphate and thrombin induce endothelium-dependent vasodilation. This response increases blood flow and inhibits the propagation of platelet thrombus. Nitric oxide also directly inhibits platelet reactivity.[10-13] Endothelium-derived NO and exogenous nitrovasodilators increase platelet cGMP, which has the effect of inhibiting platelet adherence and aggregation. There are also endothelial receptors for a number of vasoconstrictor substances such as serotonin, norepinephrine, vasopressin, and endothelin.[14,15] When the endothelial receptors are occupied by these agonists, NO is released, thereby attenuating the vasoconstriction to these agents. The endothelium tends to maintain vascular patency by halting the response to vasoconstrictors and by inhibiting platelet adherence and aggregation. In the presence of the endothelium, therefore, these vasoconstrictors cause mild vasoconstriction or even vasodilation; in the absence of a healthy endothelium, these agents act unopposed on the vascular smooth muscle, causing greater vasoconstriction. This is a homeostatic mechanism during traumatic arterial injury; the loss of endothelial influence facilitates platelet plug formation and vasoconstriction, thereby promoting hemostasis. This becomes a pathophysiologic mechanism when the endothelium is dysfunctional due to a systemic disorder (hyperlipidemia). Platelet adherence to the dysfunctional endothelium promotes thrombosis and the growth of vascular lesions.

The endothelium responds to physical forces and to humoral substances. As blood flow increases through a conduit vessel, the vessel increases in diameter.[16] This flow-mediated vasodilation requires the integrity of the

From the Section of Vascular Medicine, Division of Cardiovascular Medicine, Stanford University School of Medicine, Stanford, California.

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Reprint requests to John P. Cooke, MD, PhD, Division of Cardiovascular Medicine, Stanford University School of Medicine, 300 Pasteur Dr, Stanford, CA 94305-5246.

ABBREVIATIONS USED IN TEXT

cGMP = cyclic guanosine monophosphate
EDRF = endothelium-derived relaxing factor
MCP-1 = monocyte chemotactic protein
NO = nitric oxide
NZW = New Zealand white [rabbits]

endothelium.[17] In most vessels, flow-mediated vasodilation is largely due to the release of endothelium-derived NO, although prostacyclin, endothelium-derived hyperpolarizing factor, and other substances may contribute in some circulations.[18-20] Flow-mediated vasodilation allows the vessel to accommodate to increases in blood flow and normalizes endothelial shear stress. The ability of the endothelium to sense and to respond to changes in flow allows the vessel to respond rapidly to local changes in hemodynamics. The signal transduction mechanism by which the endothelium senses and responds to flow is still poorly understood, but the cytoskeleton and specific ionic channels likely play a role.[21,22] Long-term increases in blood flow (such as with arteriovenous fistula or with regular exercise) increase the expression of endothelial NO synthase and are associated with an enhancement of endothelium-dependent relaxation.[23,24] The effect of ongoing exercise to enhance the expression of NO synthase may explain in part the reduction in vascular events incurred by persons partaking regularly of vigorous physical activity.[25]

Nitric Oxide—A Potent Inhibitor of Platelet and Leukocyte Reactivity

Nitric oxide is not only a potent vasodilator but has substantial effects on circulating blood elements. Like other NO donors, endothelium-derived NO inhibits platelet adherence and aggregation. After being incubated in vitro with endothelial cells, human platelets are less able to aggregate.[10,13] This effect persists even after the endothelial cells are pretreated with aspirin to inhibit their production of prostacyclin, a known antagonist of platelet reactivity. This persisting inhibition of platelet aggregation is due to the effects of endothelial-derived NO because the inhibition of platelet aggregation is associated with increases in platelet cGMP, and the effects of endothelial cells can be abolished by antagonists of NO action.

It has been shown experimentally that as platelets make one circuit through the coronary microvasculature, they are "conditioned" by the endothelium.[26] Platelet-rich plasma was administered to the coronary artery perfusate of an isolated Langendorf heart preparation. Subsequently, the platelets were collected in the coronary sinus effluent. When acetylcholine was added to the perfusate to stimulate the release of endothelium-derived NO, there was a substantial increase in cGMP in the platelets traversing the coronary microcirculation. Moreover, these platelets manifested diminished aggregatory responses to adenosine diphosphate as measured by platelet aggregometry. These effects could be blocked by coinfusion of NO-synthase antagonists. The data suggest that endothelium-derived NO released by the microvascular endothelium increases platelet cGMP and diminishes platelet reactivity.

In this way, normal endothelium suppresses platelet reactivity; dysregulation of the endothelium is likely associated with a greater tendency for platelet adherence and aggregation. Indeed, preliminary studies from our laboratory indicate that platelet aggregability is enhanced in hypercholesterolemic persons; this abnormal platelet reactivity can be reversed by the oral administration of the NO precursor L-arginine.

Nitric oxide also inhibits leukocyte adherence. This effect of NO was first observed in models of ischemia-reperfusion.[27,28] When the coronary artery of an experimental animal is ligated, this induces ischemia of the myocardium served by that vessel. When the ligature is released, the ensuing reperfusion is associated with a myocardial injury that is initiated by the adherence and infiltration of neutrophils and the concomitant release of oxygen-derived free radicals. The adherence of leukocytes and subsequent reperfusion injury can be markedly inhibited by the simultaneous perfusion of the coronary artery by sodium nitrite or other exogenous NO donors.[27,28] Alternatively, reperfusing the coronary artery with solutions containing the NO precursor (L-arginine) restores endogenous NO activity and also inhibits neutrophil adherence and infiltration.[29]

A profound influence of NO on leukocyte-vessel wall interactions has been observed in other experimental models. Using videomicroscopy, the "rolling" of leukocytes may be observed along the wall of small venules. This "rolling" represents a transient adherence of the leukocyte to the vessel wall due to the interaction of adhesion molecules and their ligands on the surface of the endothelium and the leukocyte. Occasionally the leukocytes become firmly adherent and infiltrate the vessel wall. When these small vessels are perfused with antagonists of NO synthase (to inhibit the elaboration of NO), the number of leukocytes that are rolling and those that become firmly adherent is greatly increased.[30] This increased activation of endothelial-leukocyte interaction can be reversed by perfusing the vessels with the NO precursor, L-arginine. These investigations suggest that NO is a potent modulator of leukocyte-vessel wall interactions. The mechanism by which NO inhibits leukocyte adherence and infiltration is under investigation. There is evidence that NO may inhibit the expression or activity of certain adhesion molecules or chemotactic proteins (or both) involved in these processes.[31,32]

Effects on Vascular Growth

Nitric oxide also modulates the growth of vascular smooth muscle cells. In vitro, NO donors inhibit the proliferation of vascular smooth muscle cells; this effect is mimicked by the exogenous administration of 8-bromo-cGMP, a stable analogue of the second messenger of NO action.[33] Other agents such as atrial natriuretic peptide that increase the intracellular levels of cGMP also inhibit

TABLE 1.—*Antiatherogenic Interventions—Association With Nitric Oxide Activity*

Intervention	*Example*
Angiotensin-converting enzyme inhibitors	Captopril
Calcium-entry antagonists	Diltiazem, nifedipine, verapamil, felodipine
HMG CoA-reductase inhibitors	Lovastatin
Antioxidants	Probucol, superoxide dismutase
High-density-lipoprotein cholesterol infusion	- -
Diet	Fish oil, arginine

HMG CoA = 3-hydroxy-3-methylglutaryl coenzyme A

the proliferation of vascular smooth muscle cells in culture.[34] Does NO inhibit the proliferation of vascular smooth muscle cells in vivo? Some initial studies indicate that it does indeed play an important role in controlling vascular growth. In a number of disease states where the release of NO is reduced or abolished, such as restenosis, hypercholesterolemia, and hypertension, there is an increase in the proliferation of vascular smooth muscle cells within the media and the intima. Evidence from our laboratory and others indicates that by augmenting the release of endogenous NO, vascular smooth muscle proliferation can be inhibited in these disease states. By experimentally enhancing vascular NO activity (by the oral administration of arginine or by the direct transfer of the gene for NO synthase into the vessel wall), myointimal hyperplasia is reduced after balloon angioplasty in experimental models of restenosis.[35-37]

Restoration of Nitric Oxide Activity in Vascular Disease

The proliferation of vascular smooth muscle cells, monocyte adherence and infiltration, and platelet adherence and aggregation are key processes involved in atherogenesis. Because endothelium-derived NO inhibits each of these processes, we have proposed that NO is an endogenous antiatherogenic molecule.[38,39] Therefore, atherogenesis would be promoted by an endothelial injury or alteration that results in a reduction in NO activity (such as that which occurs in hypercholesterolemia).

If a reduction in NO activity promotes atherogenesis, a restoration of NO activity might be expected to retard the progression of the disease.

Endothelial Dysfunction Is Reversible

We reasoned that if we could increase the synthesis of NO by vessel walls, a number of key processes in atherosclerosis would be inhibited and progression of the disease halted. We first showed that the endothelial dysfunction induced by hypercholesterolemia could be reversed by the administration of the NO precursor, L-arginine.[40-42] New Zealand white (NZW) rabbits were fed normal chow or a high-cholesterol diet. After eight weeks, the animals received an intravenous infusion of L-arginine or an excipient (normal saline solution) over one hour. After the infusion, NO-dependent vasodilation of the hindlimb resistance vessels was stimulated by intra-arterial infusions of acetylcholine (which stimulates muscarinic receptors on the endothelium to release NO). As expected, NO-dependent vasodilation in the hypercholesterolemic animals receiving normal saline solution was more inhibited than in animals fed a normal diet. By contrast, hypercholesterolemic animals receiving the L-arginine infusion demonstrated normal NO-dependent vasodilation.[40] Subsequently, thoracic aortas were harvested from these animals for studies of NO-dependent vasodilation in vitro. Vascular rings of thoracic aortas from hypercholesterolemic animals relaxed normally to the endothelium-independent vasodilator nitroglycerin. By contrast, NO-dependent vasodilation to acetylcholine was inhibited in the hypercholesterolemic animals receiving the saline infusion, but normalized in those animals receiving the infusion of L-arginine.[41]

We confirmed these observations in hypercholesterolemic humans.[42] Previously we had demonstrated an endothelial dysfunction in the forearm resistance vessels of otherwise healthy young humans with hypercholesterolemia.[43] We assessed endothelium-dependent and endothelium-independent vasodilation of the forearm vasculature using plethysmography. Intra-arterial infusions of sodium nitroprusside increased forearm blood flow in normal and hypercholesterolemic subjects to the same degree. By contrast, vasodilation was reduced in hypercholesterolemic humans in response to intra-arterial methacholine (which stimulates the release of endothelium-derived NO). The methacholine response became normal following the infusion of L-arginine. The effects of L-arginine were likely due to its metabolism to NO, because its effects are not mimicked by D-arginine (which is not a substrate for NO synthase). We have observed similar beneficial effects of L-arginine infusions in reversing the endothelial dysfunction associated with transplantation atherosclerosis.[44] Moreover, arginine does not alter endothelium-independent responses in hypercholesterolemic animals or humans.

The restoration of endothelium-dependent vasodilation by arginine may be caused by an enhanced synthesis of NO. This implies that hypercholesterolemia induces an abnormality in the enzyme NO synthase, an impairment in arginine transport, or an alteration in arginine metabolism by other intracellular pathways. Alternatively, arginine may have an indirect effect (such as reducing NO degradation by superoxide anion). This last mechanism, however, would not explain our observation that the ex vivo generation of nitrogen oxides is increased in the thoracic aorta of hypercholesterolemic animals receiving dietary arginine (discussed later).

Evidence That Nitric Oxide Inhibits Atherogenesis

Having shown that vascular NO activity could be normalized in persons and animals with hypercholesterolemia, we then postulated that a sustained improve-

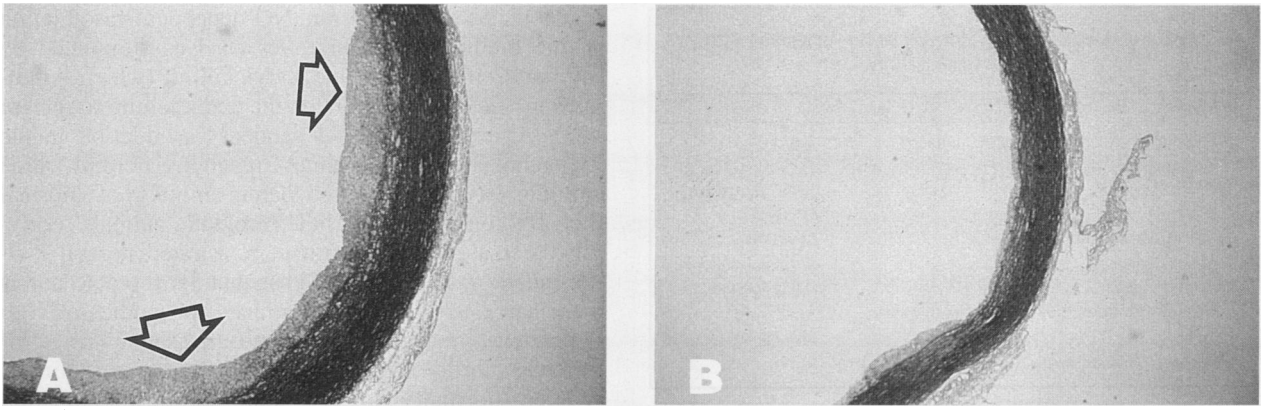

Figure 1.—After 10 weeks of a 0.5% cholesterol diet, 40% of the surface area of the thoracic aorta of New Zealand white rabbits was involved by intimal lesions (**arrows; A**). In contrast, in animals fed a high-cholesterol diet supplemented with dietary arginine, surface area of the lesions was reduced by 75% (**B**) (from Cooke et al[38]).

ment in NO release would inhibit atherogenesis.[38,45] This hypothesis was supported indirectly by previous observations that all known agents that suppressed atherogenesis also enhanced NO-dependent vasodilation, where this had been assessed (Table 1). To test this hypothesis, we placed NZW rabbits on a normal or high-cholesterol diet; some of the animals on the high-cholesterol diet also received a sixfold increase in dietary arginine (or methionine, to control for changes in nitrogen and caloric balance). The supplemental dietary arginine did not alter the lipid profile nor any hemodynamic values in the hypercholesterolemic animals; the only difference between the hypercholesterolemic groups was that plasma arginine levels were doubled in the animals receiving dietary arginine. At ten weeks, the thoracic aorta and coronary arteries were harvested for studies of vascular reactivity and histomorphometry. In vitro, the contractions to norepinephrine were not different between groups (there are no adrenergic receptors on the endothelium of rabbit thoracic aortas; the effect of norepinephrine on the vessel wall is mediated solely by α_1-adrenergic receptors on the vascular smooth muscle). There was also no difference between groups in endothelium-independent relaxations to sodium nitroprusside. As expected, NO-dependent vasodilation to acetylcholine was inhibited in the hypercholesterolemic animals receiving the excipient. By contrast, hypercholesterolemic animals receiving L-arginine had an improvement in NO-dependent vasodilation to acetylcholine.

The improvement in vascular NO activity had a striking effect on vascular structure. Lesion surface area was reduced by about 75% in the thoracic aortas from hypercholesterolemic animals (Figure 1).[38] In the coronary arteries, lesions were observed in all hypercholesterolemic animals receiving the excipient, but were pristine in those hypercholesterolemic animals receiving arginine.[45]

We are investigating the mechanism of this striking antiatherogenic effect of NO. It appears to be due in part to the effect of endogenous NO to inhibit monocyte adherence to the endothelium in hypercholesterolemic animals.[46] We have observed that after only two weeks of a high-cholesterol diet, the thoracic aorta of hypercholesterolemic animals has increased adhesiveness for mononuclear cells, as demonstrated by a functional binding assay in vitro. Segments of thoracic aorta are placed in culture medium and incubated with monocytoid cells or with mononuclear cells isolated from the blood of normocholesterolemic rabbits. After 15 minutes, nonadherent cells are washed away with fresh medium and the number of bound cells enumerated using videomicroscopy. Substantially more monocytes adhere to the thoracic aortas from hypercholesterolemic rabbits (a threefold increase in the number of adherent cells). The number of adherent cells is reduced in thoracic aortas from hypercholesterolemic animals receiving dietary arginine. This is associated with an increase in the release of NO from these tissues. By contrast, when nitroarginine (an antagonist of NO synthase) is administered to animals with normal cholesterol levels, the number of adherent cells is dramatically increased. Thus, reductions in NO activity by hypercholesterolemia, or the inhibition of NO synthesis, is associated with increased monocyte-endothelial cell binding.

This effect of NO on endothelial adhesiveness for monocytes is likely due to its inhibition of adhesive glycoproteins or chemokines mediating endothelial-monocyte interaction. Accumulating evidence indicates that hypercholesterolemia imposes an oxidative stress on endothelial cells. This alteration in the endothelial redox state activates specific transcriptional pathways that induce the expression of oxidant-responsive genes.[47] These genes encode proteins such as vascular cell adhesion molecule (VCAM-1) and monocyte chemotactic protein (MCP-1), molecules that mediate monocyte binding to the endothelium. We postulate that endothelium-derived NO interferes with the activation of these transcriptional pathways by modulating the endothelial redox state. Nitric oxide is known to suppress the elabo-

ration of oxygen-derived free radicals by neutrophils, probably by inactivating oxidative enzymes that generate superoxide anion.[48] Nitric oxide likely exerts an inhibitory effect as well on endothelial oxidative enzymes. We and others have found that hypercholesterolemia causes endothelial cells to generate superoxide anion[49]; this effect of hypercholesterolemia can be mimicked by antagonism of NO synthase[50] and can be suppressed by exogenous NO donors or by the enhancement of endogenous NO activity.[51] Our preliminary studies in cell culture and in vivo reveal that the effect of NO to suppress endothelial generation of superoxide anion is associated with a decrease in NFκB-mediated expression of MCP-1 and a parallel suppression of monocyte binding to the endothelium.

Regression and Progression—Dependency on Nitric Oxide

At this point, we had found that by enhancing vascular NO activity we could suppress the development of atherogenesis. An important question remained unanswered, however: in the presence of preexisting endothelial dysfunction and vascular disease, can the administration of arginine restore NO activity and slow the progression of disease (or even induce regression)?

We have performed preliminary studies to address this question.[51] We administered normal or high-cholesterol chow to NZW rabbits for ten weeks. At this time, 30% to 40% of the thoracic aorta is involved by lesions, and there is reduced NO activity, as manifested by attenuated endothelium-dependent relaxations. Arginine was then added to the 0.5% cholesterol diet of half of the animals. At 10, 14, 18, and 23 weeks into the experimental protocol, the thoracic aorta was harvested for studies of vascular reactivity and histomorphometry. Administering arginine restored NO activity in most animals at 14 and 18 weeks.[52] This effect was associated with an apparent regression of disease. By contrast, hypercholesterolemic animals receiving the excipient had a progression of disease. By 23 weeks, administering arginine was no longer enough to restore NO activity in most animals receiving supplementation: in these animals, lesion surface area progressed. In a few animals, however, administering dietary arginine maintained vascular NO activity at normal levels; these "responders" enjoyed continued regression. Preliminary studies indicate that these effects of NO may be mediated by the suppression of redox-sensitive transcriptional pathways activating MCP-1.

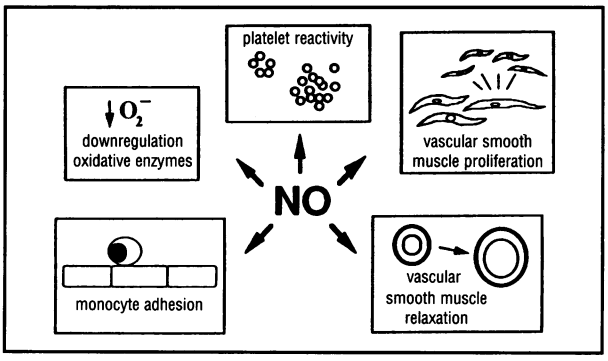

Figure 2.—Nitric oxide (NO) is an endogenous antiatherogenic molecule. In addition to being a potent vasodilator, it may inhibit a number of key processes in atherogenesis, including vascular smooth muscle proliferation, platelet adherence or aggregation, the generation of oxygen-derived free radicals, and monocyte adherence and infiltration (possibly by inhibiting the expression or activity of endothelial adhesion molecules or chemotactic proteins).

To conclude, restoring NO activity reduced monocyte adhesion and accumulation in the vessel wall. The explanation for the apparent regression remains undetermined, but may include either or both an effect of NO on the elaboration of chemokines and adhesion molecules mediating the influx of monocytes or the efflux of lipid-laden macrophages. Apoptosis of cells contributing to lesion growth is another mechanism by which NO could mediate lesion regression.

Summary

The cumulative data indicate that NO is an endogenous antiatherogenic molecule by virtue of its effects to inhibit monocyte adherence, platelet aggregation, and vascular smooth muscle proliferation (Figure 2). These salutary effects of NO may be mediated by its effect in modifying the endothelial redox state, as well as increasing intracellular cGMP levels. The impairment of NO activity by hypercholesterolemia, diabetes mellitus, or hypertension likely plays a role in the initiation of atherosclerosis. Recent insights into endothelial biology suggest a new paradigm for atherosclerosis, where modulation of the endothelial redox state plays a major role in the expression of genes related to atherogenesis. It is likely that these basic insights regarding mechanisms of atherogenesis will lead to new therapeutic strategies to halt the progression, or induce the regression, of atherosclerosis.

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